

(12)

**EUROPEAN PATENT APPLICATION**

(21) Application number: 85308827.6

(51) Int. Cl.<sup>4</sup>: **C 12 N 15/00**

(22) Date of filing: 04.12.85

(30) Priority: 05.12.84 US 678578

(43) Date of publication of application:  
11.06.86 Bulletin 86/24

(84) Designated Contracting States:  
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: **ALLELIX INC.**  
6850 Goreway Drive  
Mississauga Ontario, L4V 1P1(CA)

(72) Inventor: **Buxton, Francis Paul**  
727 Indian Road  
Toronto Ontario, M6P 2E3(CA)

(72) Inventor: **Gwynne, David Ivor**  
34 Monterrey Avenue  
Brampton Ontario(CA)

(72) Inventor: **Davies, Roger Wayne**  
R.R. No. 1  
Limehouse Ontario L0P 1H0(CA)

(74) Representative: **Perry, Robert Edward et al,**  
**GILL JENNINGS & EVERY** 53-64 Chancery Lane  
London WC2A 1HN(GB)

(54) **Transforming aspergillus niger, and plasmids for use therein.**

(57) Transformants of *Aspergillus niger* and related *Aspergillus*, containing foreign DNA conferring modified properties of expression thereon, are prepared by use of a DNA vector which contains a selectable marker which is capable of incorporation into the DNA of the host *A. niger* cells, but which is not to be found in the *A. niger* cells prior to this transformation. The vector also contains other foreign DNA to be incorporated into the *A. niger*, for modified expression. The process suitably uses mutants of *A. niger* as hosts, the mutants lacking the selectable marker as compared with wild-type *A. niger*, and conducts the transformation on spheroplasts of *A. niger*.

This invention relates to fungal transformants, and to methods of effecting transformation of fungi. More specifically, it relates to transformation of the filamentous fungal species Aspergillus niger and related Aspergilli, intermediates for use therein, and novel, useful fungal transformants thereof produced by genetic engineering techniques.

The fungal species Aspergillus niger is used widely in the industrial production of enzymes, e.g. for use in the food industry. Its use is based on the secretory capacity of the microorganism. A. niger is used in large quantities and on a commercial scale for this industrial production, and is consequently a well-characterized micro-organism. To apply genetic engineering techniques to A. niger, it is necessary to transfer DNA into A. niger so that the resultant fungal transformant may express an additional useful product and secrete it in large amounts.

Numerous genes have been cloned in a variety of prokaryotic vectors, and efforts are being made to obtain high expression levels for the encoded proteins. Genes are likely to be more efficiently expressed and processed and to produce a product more nearly the same as the original protein if these genes are placed into a host that is the same as or closely related to the species from which the gene was originally extracted.

However, very high levels of expression of individual proteins can be deleterious, if not lethal, to the cell if

allowed to build up to high concentrations therein, and can cause crystallization of the protein into inactive and highly insoluble inclusion bodies. Thus one should develop systems that allow efficient excretion of synthesized proteins from the cell. Also, it is best to choose as hosts organisms which have been grown commercially, so that the already well developed fermentation technology and downstream processing can be exploited in the production of novel products or the increased production of existing products. A. niger is the source organism for numerous industrially important proteins, enzymes and other products. It is also capable of excreting highly expressed proteins. The transformation of filamentous fungi such as A. niger has however proved particularly difficult.

In accordance with the present invention, transformants of the fungal species Aspergillus niger (or related Aspergilli) are prepared, which contain foreign DNA conferring modified properties of expression on the Aspergillus niger, and having a rapidly selectable phenotype permitting them to be readily differentiated from the original Aspergillus niger.

In the process of the present invention, Aspergillus niger is transformed by use of a DNA vector which contains a selectable marker which is capable of incorporation into the DNA of the host A. niger cells, but which is not to be found in the A. niger cells prior to this transformation. The vector may also contain other foreign DNA sequences required to be introduced into the A. niger, to enhance or modify its useful

expressions of proteins. Then the transformants so formed can be selected and isolated from the unchanged cells on the basis of the selectable marker incorporated therein, grown and cultivated in the usual way, to provide colonies of transformants of A. niger with enhanced or modified capabilities of useful protein expression.

In the Accompanying Drawings:

Figure 1 of the accompanying drawings is a diagrammatic representation of the process for preparing vector plasmid pDG1 for use in preparing A. niger transformants in accordance with the present invention;

Figure 2 is a similar diagrammatic representation for preparing alternative vector plasmid pDG3.

The selectable marker used in the present invention is suitably one which is naturally present in A. niger, so that its presence in the transformants will not materially affect the properties thereof as compared with the wild type A. niger. Thus, in the preferred process of the invention, a mutant strain of A. niger is used as the host for transformation, the mutant being one which lacks the chosen genetic marker as compared with the wild type A. niger. The mutant is transformed with a vector containing the selectable marker and the foreign DNA required to be incorporated, to modify or enhance the properties of expression of A. niger.

The use of a mutant A. niger strain to be transformed,

coupled with a vector plasmid capable of complementing the A. niger mutant host, allows for straightforward selection and isolation of successfully transformed cells from unchanged host cells. It enables one to avoid reliance for selection on the other foreign DNA introduced by the vector plasmid which, although perhaps containing the gene which it is primarily desired to incorporate for its properties of expression in A. niger, may not provide the basis of easy and rapid selection and separation of the transformants.

In occasional cases, where the desired modification to A. niger is to introduce DNA sequences which will express a specific protein not normally present in A. niger, and which has an easy assay, these sequences themselves might constitute a dominant selectable marker, and hence permit the use of wild type A. niger as host. However, such instances are rare.

In specific applications, it may be necessary to avoid having to obtain a mutant in the desired host. To transform a wild type A. niger, one can use a dominant selectable marker, i.e. a gene which specifies a novel phenotype such as ability to utilise a metabolite that is not usually metabolized by A. niger, or ability to resist toxic effects of a chemical or antibiotic. Transformants of the wild type A. niger can then be selected on the basis of the dominant selectable marker introduced therein.

A specific preferred example of a selectable marker is the Arg B<sup>+</sup> gene coding for the enzyme ornithine transcarbamylase. This enzyme is present in wild type A. niger. Mutants lacking this enzyme (Arg B<sup>-</sup> strains) can be

prepared by usual non-specific techniques, e.g. treatment with ultraviolet radiation, and selected by their ability to grow on a medium containing arginine, but not on minimal medium.

Transformants made according to the process of the invention, from an Arg B<sup>-</sup> strain of A. niger and a vector containing the Arg B<sup>+</sup> gene, are consequently Arg B<sup>+</sup> and are readily selectable and isolatable from the non-transformed Arg B<sup>-</sup> strain, by standard plating out and cultivation techniques.

Whilst it is preferred, as described above, to utilize a selectable marker which is natural to wild type A. niger, it is not in fact essential that the selectable marker as used in the vector be actually derived from A. niger. It can equally well be obtained from another, similar species which also contains the required gene, subject to limitations of expression. Thus, in the preferred case under the present invention, the selectable marker Arg B<sup>+</sup> can be obtained by treatment of fungal DNA of species Aspergillus nidulans with appropriate restriction enzymes, and then ligated into a suitable vector plasmid for use in transforming A. niger (Arg B<sup>-</sup>).

In the preferred embodiment of the process of the present invention, spheroplasts of A. niger are prepared and used in the transformation. The preferred method of preparing the spheroplasts is by enzymatic digestion of the cell walls, using for example cellulases. The selection of a suitable enzyme for enzymatic digestion of A. niger to produce spheroplasts thereof is within the skill of the art, perhaps

requiring some routine screening tests. Useful enzymes are those capable of digesting complex polysaccharides, and are found among those known as effective in preparing fungal spheroplasts of a wide variety of fungal species. Specific examples of suitable enzymes include Novozym 234 (an impure mutanase) and B-glucuronidase. Other suitable methods may be used to form spheroplasts. Moreover, with suitable methods for cell wall penetration by the vectors, whole cells of A. niger may be used, instead of spheroplasts.

Transformations according to the present invention are suitably conducted using an appropriately chosen vector plasmid. To be suitable for use herein, the vector must contain the chosen selectable marker (e.g. Arg B<sup>+</sup>), and it must contain the other useful gene which the transformants are to contain. The vector may comprise linear or circular DNA. Preferably the vector also contains an E. coli replicon so that it can be manipulated and replicated therein, for introduction of selectable markers and the like, and preparation of adequate quantities of the vector in E. coli.

In a specific embodiment, the present invention provides a transformation system that allows selection of arginine prototrophic colonies from an Aspergillus niger arginine auxotroph (arg B<sup>-</sup> 350.52) by treating spheroplasts, typically obtained by enzymatic (for example Novozym 234 and B-glucuronidase) digestion of the cell wall followed by subsequent purification, with DNA, in the form of a plasmid, that contains the ornithine transcarbamylase structural gene of

0184438

A. nidulans in the presence of polyethylene glycol 4000 and  $\text{CaCl}_2$ . These transformants integrate the arg B<sup>+</sup> piece of DNA into their chromosomes and are capable of expressing this gene. The transformants also take up the other DNA present in the plasmid, shown by the presence of sequences in the transformants that hybridize to pBR322 and Tn5 that are not present in the parental strain.

Thus, in summary, to perform the transformation described in this invention one needs a recipient strain and a piece of DNA of which a part is selectable in this strain, i.e. contains a selectable marker. The strain should be of the species Aspergillus niger or closely related species of the Aspergillus genera. However, the recipient strain and the transforming DNA have to be chosen so that the transformants obtained by the uptake of the DNA into the recipient have a readily selectable phenotype. Thus one can use recipients that are sensitive to toxic metabolites with a DNA that encodes resistance (e.g. G418 and Tn5) or recipients that are unable to grow on a particular source of carbon, nitrogen etc., with a DNA that encodes the ability to use this (e.g. acetamide and acetamidase), or use prototrophic recipients and a DNA that encodes the missing enzyme.

The invention is illustrated in the following specific examples.

Aspergillus niger (ATCC 46951) was mutagenized with UV light and an isolate obtained that required ornithine or arginine for growth in a defined minimal media. This strain



which lacks ornithine carbamoyl transferase was called arg B (350.52). Media for growing Aspergillus niger were as described for A. nidulans by Cove, Biochem Biophys Acta (1966) 113, 51-56.

#### Recombinant DNA

Tn5 is described in Berg et al "Biotechnology" (1983) 1, 417-435; pHC79 is described in Hohn et al "Gene" (1980) 11, 291-296; pUC12 is described in Vieira et al "Gene" (1982) 19, 259-268; and pBB116 described in Berse et al "Gene" (1983) 25, 109-117.

Restriction enzymes were obtained from International Biotechnologies Incorporated and were used as per manufacturers instructions. Plasmids were prepared by the alkaline lysis procedures of Ish-Horowitz et al "Nucleic Acids Research" (1981) 9, 2989-2998.

The preparation and construction of two novel vector plasmids, useful in the process of the present invention, is diagrammatically illustrated in the accompanying drawings. In Figure 1, the process utilizes the Arg B<sup>+</sup> gene extracted by known techniques using the enzyme Bam H1 from DNA of A. nidulans. This fraction is initially ligated with known E. coli plasmid pBB8 using ligase to form plasmid pBB116, which is also known. Then plasmid pBB116 is treated with restriction enzymes EcoRI and Bam H1 to prepare fragments thereof. The fragment containing the Arg B<sup>+</sup> gene may be isolated from the mixture by normal methods, e.g. agarose electrophoresis and electroelution, and purified. Then it is ligated with known E. coli plasmid pHC79 also cut with Bam H1 and EcoRI, to produce novel plasmid

pDG1. If desired, isolation of the fragment may be omitted, the ligation step performed using the entire mixture, and the plasmids containing the desired sequences then selected by known methods. This novel plasmid contains the replication origin, Ori, derived from pHC79 permitting it to be produced in quantity by replication in E. coli. It contains the Arg B<sup>+</sup> gene as a selectable marker in Aspergillus, and has other DNA sequences, which can be introduced by transformation by known techniques into spheroplasts of A. niger Arg B<sup>-</sup> mutants. The resulting transformants can be selected on the basis of their Arg B<sup>+</sup> characteristic, from the non-transformed mutants, and isolated and cultivated.

Figure 2 diagrammatically illustrates a construction of alternative plasmid pDG3 for effecting transformation of A. niger strains Arg B<sup>-</sup>. In this process, known E. coli plasmid pUC 12 is ligated with a portion of the known transposon Tn5, specifically that portion containing the kanamycin resistance gene (Kan<sup>R</sup>), using restriction endonucleases Hind III and Sal I and T4 ligases. The resultant plasmid vector, pNE02, is then cut with XbaI and ligated with pBB116 XbaI fragments to produce plasmid pDG3. This novel plasmid pDG3 is approximately 7.4 kilo-base pairs long, and contains the Kan<sup>R</sup> gene derived from Tn5, the Amp<sup>R</sup> gene derived from pUC12 and the Arg B<sup>+</sup> gene derived from pBB116. It can be used in transformations with spheroplasts of Arg B<sup>-</sup> mutants of A. niger.

Both pDG 1 and pDG 3 contain in their DNA sequences both the selectable marker Arg B<sup>+</sup> and other DNA foreign to A.

niger for incorporation into A. niger. This foreign DNA can include useful genes conferring novel and beneficial properties on A. niger transformants.

It is to be noted in connection with the present invention, that the vector comprising the foreign DNA is integrated into the genome of the recipient cell, i.e. it becomes part of the chromosomal or nuclear DNA, rather than remaining as a plasmid, so that the expression of the foreign DNA is thereafter permanent and there is little chance of its subsequent loss from the transformed cell. The novel phenotype of the transformants is thus very stable.

#### EXAMPLE 1 - TRANSFORMATION OF ASPERGILLUS NIGER

500 mls of complete media (Cove 1966) + 0.02% arginine +  $10^{-5}$ % biotin in a 2 l conical flask was inoculated with  $10^5$  conidia/ml of A. niger strain 350.52 (arg B) prepared as previously described, and incubated at 30°C, shaking at 250 rpm for 20 hours. The mycelia was harvested through Whatman No. 54 filter paper, washed with sterile deionized water and sucked dry. The mycelia was added to 50 ml of filter sterile 1.2 M  $MgSO_4$  10 mM potassium phosphate pH 5.8 in a 250 ml flask to which was added 20 mg of Novozym 234 (Novo Enzyme Industries), 0.1 ml (=15000 units) of  $\beta$ -glucuronidase (Sigma) and 3 mg of Bovine serum albumin for each gram of mycelia. Digestion was allowed to proceed at 37°C with gentle shaking for 50-70 minutes checking periodically for spheroplast production by light-microscope. 50 mls of sterile deionised water was added

0184438

and the spheroplasts were separated from undigested fragments by filtering through 30  $\mu$ m nylon mesh and harvested by centrifuging at 2500 g for 5 minutes in a swing out rotor in 50 ml conical bottom tubes, at room temperature. The spheroplasts were washed, by resuspending and centrifuging, twice in 10 mls of 0.6 M KCl. The number of spheroplasts was determined using a hemocytometer and they were resuspended at a final concentration of  $10^8$ /ml in 1.2 M Sorbitol, 10 mM Tris/HCl, 10 mM  $\text{CaCl}_2$  pH 7.5. 0.4 ml aliquots were placed in plastic tubes to which DNA either pDG 1 or pDG 3 in different experiments, (total vol. 40  $\mu$ l in 10 mM Tris/HCl 1 mM EDTA pH 8) was added and incubated at room temperature for 25 minutes. 0.4 ml, 0.4 ml then 1.6 ml aliquots of 60% PEG4000, 10 mM Tris/HCl, 10 mM  $\text{CaCl}_2$  pH 7.5 were added to each tube sequentially with gentle, but thorough mixing between each addition, followed by a further incubation at room temperature for 20 minutes. The transformed spheroplasts were then added to appropriately supplemented minimal media 1% agar overlays, plus or minus 0.6 M KCl at 45°C and poured immediately onto the identical (but cold) media in plates. After 3-5 days at 37°C the number of colonies growing was counted.

Transformants which have successfully taken up the Arg  $\text{B}^+$  and other DNA from the vector plasmids pDG 1 and pDG 3 will grow on minimal media plus KCl - the potassium chloride being necessary to prevent rupture of the spheroplasts.

The results are given in the following Table.

DNA Added			No. of transformants growing on minimal +KCl, after 6 days
Type	vol( l)	wt( g)	
none	0	0	0
pDG 1	40	20	12
pDG 3	40	80	300

All viable cells and spheroplasts, including unchanged cells, unchanged spheroplasts, transformants, and revertants, will grow on minimal media plus arginine plus KCl. Such growth medium serves to check on the percentage survival of viable material, which was in these experiments in the range 40-90%. On minimal media plus arginine, only contaminating, non-spheroplast materials will grow. The contamination with such non-osmotically sensitive cells was less than 1% in the present experiments.

#### Example 2

To check if these are genuine transformants rather than revertants, transformants were grown in minimal media, DNA was prepared from them, and they were probed with radioactively labelled plasmids.

Mycelia, grown from 36 to 72 hours from an inoculum of  $10^5$  conidia/ml in 50 mls of appropriately supplemented media, were harvested through Whatman No. 54 paper and washed with

0184438

distilled water. The mycelium was transferred to a cold mortar and ground to a fine powder in approximately 50 mls of liquid nitrogen with a pestle. This powder was poured into a 50 ml sterile siliconized conical flask to which was added 5 mls of 50 mM ethylenediaminetetraacetic acid, 0.2%, sodium dodecyl sulfate pH 8.5 and 5  $\mu$ l of diethyl pyrocarbonate. This suspension was shaken for 1 minute at room temperature, then heated at 68°C for 20 minutes. The cellular debris was removed by centrifuging the suspension in a 15 ml Corex tube at 4°C X 8000 rpm X 15 minutes in a Sorvall SS34 rotor. Using wide mouthed Gilson tips 4 mls of the clear supernatant was removed to a fresh 15 ml Corex tube. To this was added 0.25 mls of 8 M acetic acid/KOH pH 4.2. This solution was incubated on ice for 45 minutes and the resulting precipitate was removed by centrifuging at 16000 rpm X 15 min X 4°C in a Sorvall SS34 rotor. 3 mls of the clear supernatant was transferred as before to 3 mls of propan-2-ol in a fresh 15 ml Corex tube. After mixing, the DNA was pelleted by centrifuging at 10000 rpm X 4 min X 4°C in a Sorvall SS34 rotor. The drained pellets were redissolved in 0.5 ml of 10 mM Tris/HCl 1 mM EDTA pH 7.5 by gentle agitation. This solution was transferred to 1.5 ml microfuge tubes and 1  $\mu$ l of a 10 mg/ml stock of boiled RNAase was added. The RNA was digested at 37°C for 20 minutes.

Then 60  $\mu$ l of 3 M sodium acetate pH 6 and 660  $\mu$ l of propan-2-ol was added. The precipitated DNA was pelleted by centrifuging at 15000 g for 2 mins. The pellet was dried briefly in a Speed-vac and then redissolved in 100  $\mu$ l of 10 mM

Tris/HCl 1 mM EDTA pH 8.

0184438

Southern transfers and hybridizations were as described in Buxton et al "Molecular General Genetics" (1983) 190:403-405. DNA for dot blots was denatured in 9 volumes of 1M NaOH for 10 min and neutralized with a further 1.5 volume of 0.7M Tris/HCl, 0.3M NaCl, 20mM EDTA, pH 6.8. After application to nitrocellulose using a Bethesda Research Laboratories's dot blot apparatus the filters were treated as for Southern blots.

DNA from 8 transformants, A. niger strain 350.52 and A. nidulans FGSC4, were loaded onto dot-blots and probed with pBR322. Sequences homologous to pBR322 were detected in 6 of the 8 transformants, but not in either A. nidulans wild type or in the A. niger recipient from which these transformants were derived. A Southern blot was also done of BamHI digested DNA of these strains and probed with pDG1. A. nidulans wild type showed one BamHI fragment homologous to the arg B gene. Under the conditions of washing, A. niger 350.52 showed no bands hybridizing. Five of the transformants showed discrete bands, the same pattern not being observed in different transformants either indicating different integration events at the same locus or integration at different sites in the genome. Transformants obtained with pDG 3 also showed hybridization to Tn 5 sequences indicating the uptake of Kan<sup>R</sup> gene from pDG 3.

Thus, it is concluded that A. niger (strain 350.52) arg B<sup>-</sup> has been transformed with the A. nidulans arg B<sup>+</sup> gene. This gene is expressed in A. niger. The growth rates of transformants are not very good, so it is probably rather poorly

expressed. In 75% of cases, the vector is also integrated into the genome along with the arg B<sup>+</sup> gene in these transformants, so that a viable method of transforming foreign DNA into A. niger is provided.

5       The stability of the transformed phenotypes thus prepared was checked. Transformants were inoculated from minimal media on to complete media and 10 mM arginine, and grown for 5 days from a central inoculum. Conidia were removed from the edge of the colony, diluted  
10       appropriately, and plated on to minimal plus 10 mM arginine. After 3 days, colonies were replica plated on to minimal and minimal plus arginine. Out of 400 colonies tested from 8 transformants, all were prototrophic. This indicates that the transformed  
15       phenotype is perfectly stable over this growth period, as would be expected for any gene integrated into the genome.

      The following viable cultures were deposited with the American Type Culture Collection on 24th January  
20       1985: E. coli HB101 containing pDG1 and E. coli JM83 containing pDG3, designated respectively ATCC 53005 and ATCC 53006, and Aspergillus niger strain 350.52, designated ATCC 20739.

25

30

35



CLAIMS

1. A process for preparing transformants of the fungal species Aspergillus niger and related Aspergilli, which comprises treating an A. niger strain lacking a  
5 selectable marker with a DNA vector containing said selectable marker and other DNA sequences, under conditions permitting at least some of the A. niger cells to take up the DNA vector.
2. A process according to claim 1, wherein spheroplasts  
10 of the A. niger strain are treated with the DNA vector.
3. A process according to claim 1 or claim 2, wherein the DNA vector is circular.
4. A process according to claim 1 or claim 2, wherein the DNA vector is linear.
- 15 5. A process according to any preceding claim, wherein the vector includes a replicon, permitting its replication in a bacterial host.
6. A process according to any preceding claim, wherein the selectable marker is characteristic of  
20 naturally-occurring, wild-type A. niger, and the A. niger strain which is treated with the vector is a mutant strain thereof, deficient in said selectable marker.
7. A process according to claim 6, wherein the selectable marker is antibiotic resistance.
- 25 8. A process according to claim 6, wherein the selectable marker is a gene for utilisation of metabolites not usually utilised in A. niger.
9. A process according to any preceding claim, wherein the selectable marker is a gene producing an  
30 easily-assayable product.
10. A process according to any preceding claim, wherein the selectable marker is the ornithine carbamoyl transferase gene.

11. A process according to claim 10, wherein the ornithine carbamoyl transferase gene is derived from A. niger or A. nidulans.

12. A process according to any preceding claim, wherein  
5 the vector is the plasmid pDG1 or pDG3 as herein described.

13. A process according to any preceding claim, which comprises the additional step of cultivating the transformants.

10 14. The plasmid pDG1 or pDG3 as herein described.

15

20

25

30

35

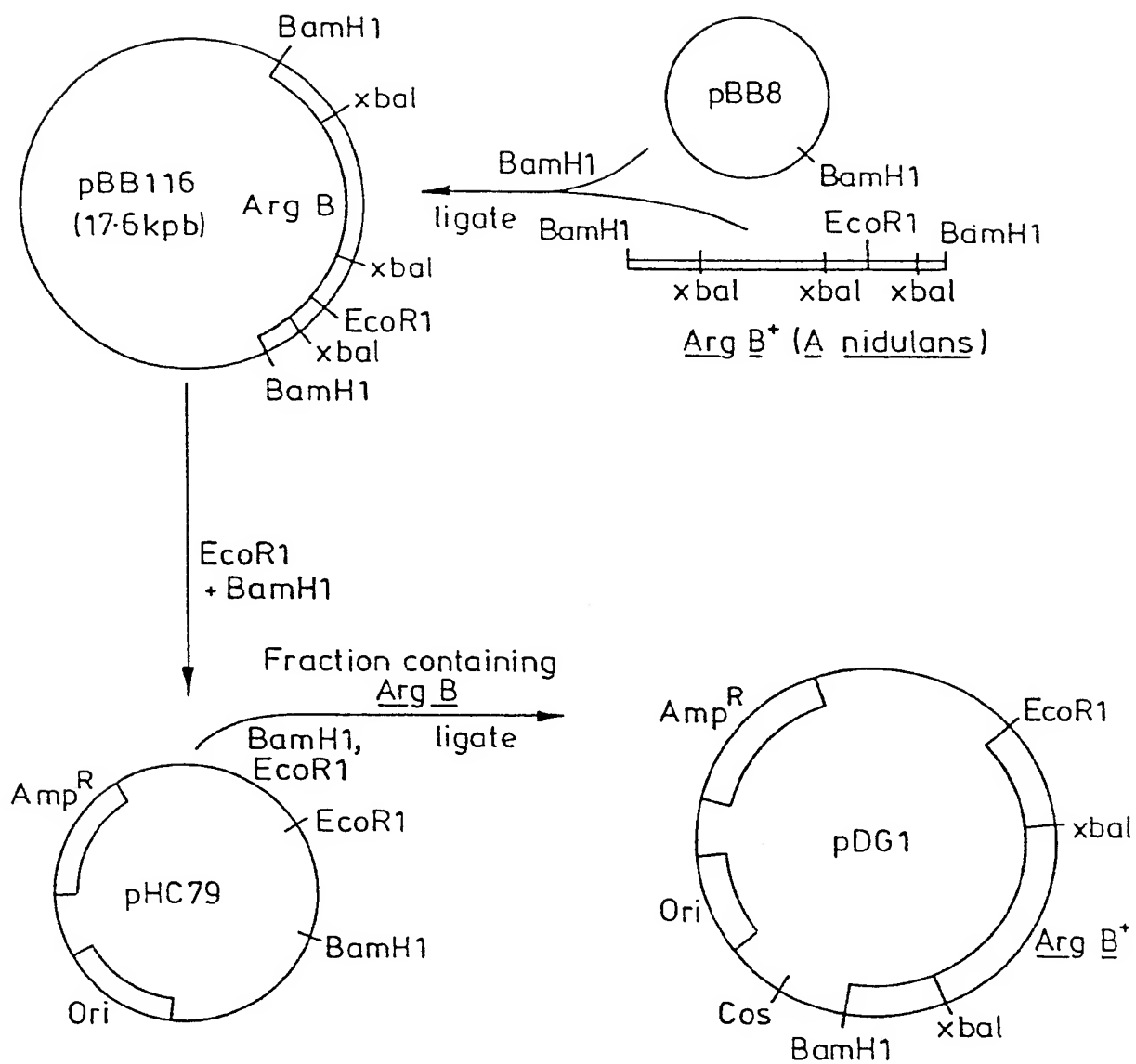


FIG. 1

2/2

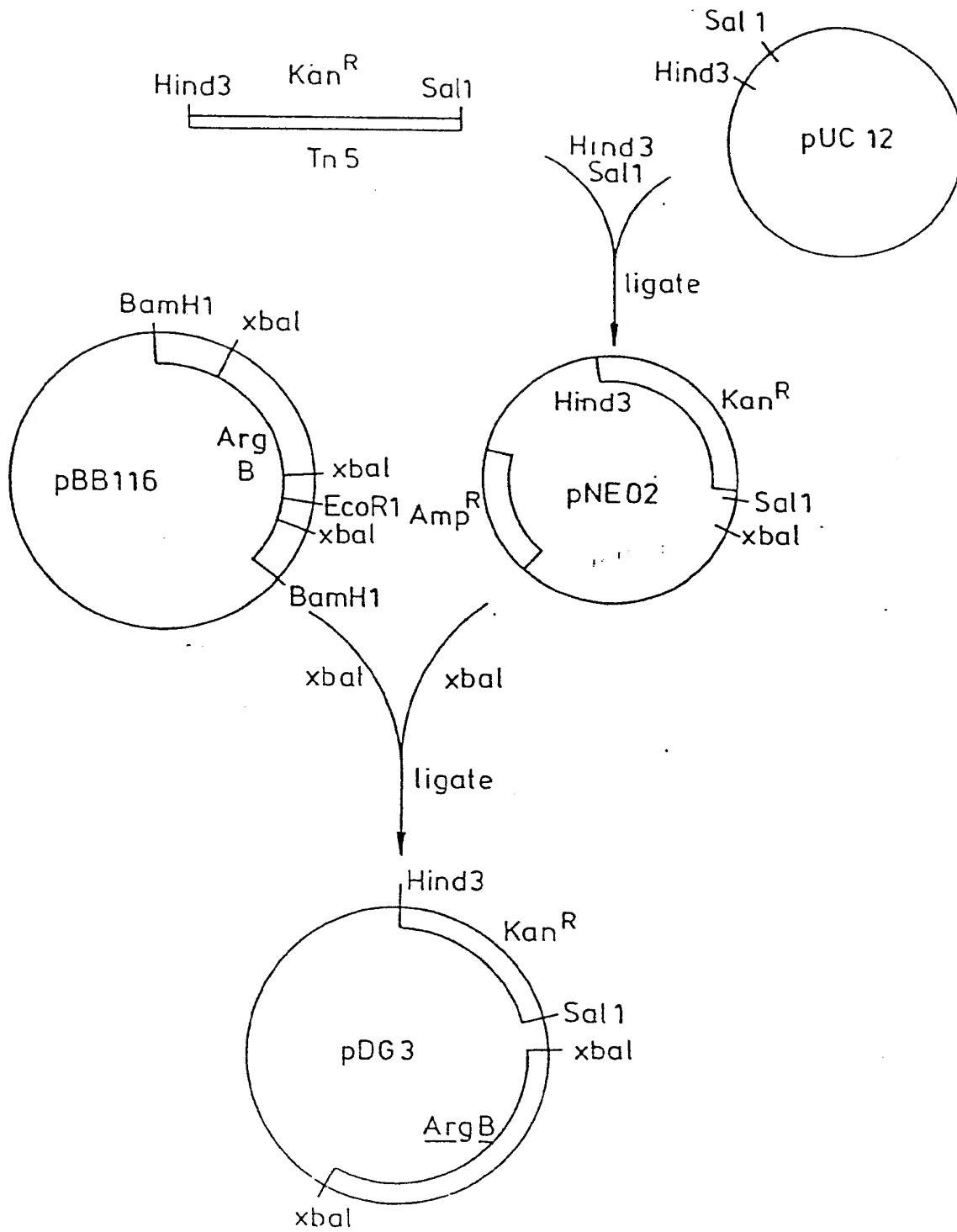


FIG. 2